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Development of a Sperm Cell Toxicity Test for Marine Waters

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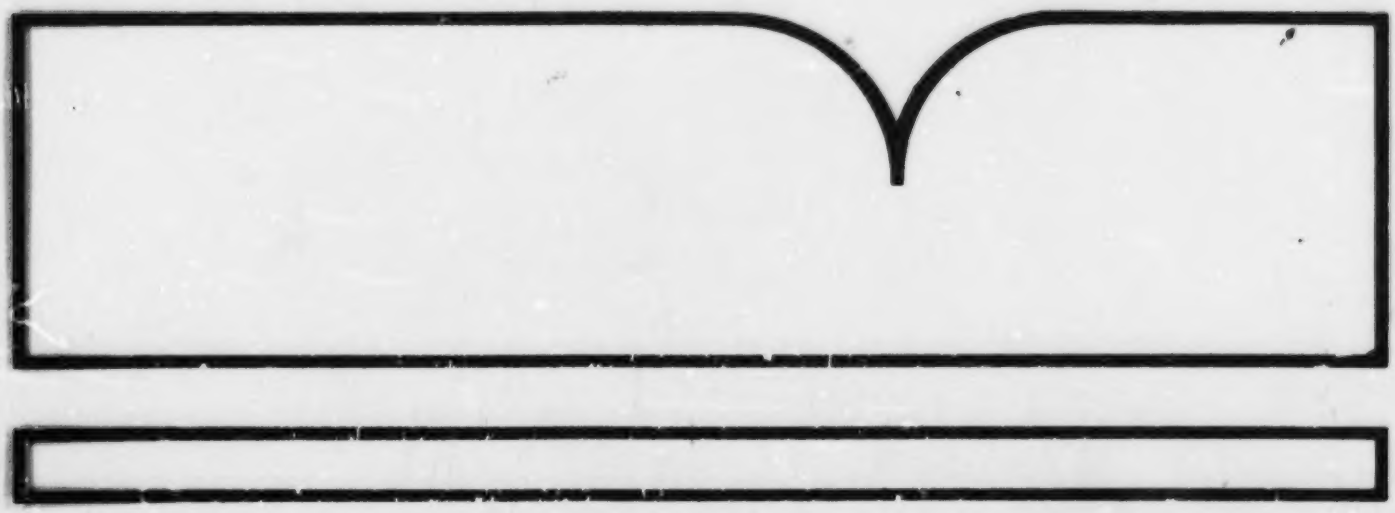

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Sperm cell toxicity tests with two reference toxicants (silver nitrate and the pesticide endosulfan) showed that toxic responses were directly related to sperm exposure time. The 60 min. EC_{50}s and 95% fiducial limits for green sea urchin (Stronglyocentrotus droebachiensis) sperm were 76 (57 to 103) and 168 (99 to 285) g/litre for silver and endosulfan, respectively. The 60 min. EC_{50}s for sand dollar (Dendraster excentricus) sperm were 45 (34 to 61) and 147 (123 to 175) g/litre for silver and endosulfan, respectively. Matrix bioassays using temp., salinity, and toxicant concentration as variables showed that the toxic response to both silver and endosulfan generally increased with increasing temp. and decreasing salinity. Comparative sea urchin and sand dollar embryo tests (72 to 96h) showed embryo sensitivity to be similar to sperm sensitivities with levels of effect (retardation of development) of 50 and 25 g/litre, respectively, for silver and 253 and 158 g/litre for endosulfan.

Additional development and refinement of this sperm cell toxicity test should yield an additional tool for the biomonitoring of receiving water quality.

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Development of a Sperm Cell Toxicity Test for Marine Waters

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ABSTRACT: Preliminary methods for conducting a quick and sensitive sperm cell toxicity test for marine waters have been developed. This paper presents a simple static test in which sea urchin or sand dollar sperm cells are exposed to test or control solutions for short periods of time (typically 15 to 60 min). Eggs are added to the test containers after the sperm exposure period has ended, and a standard time is allowed for fertilization to take place before the eggs are fixed with formalin. Samples are then tabulated for fertilization success by noting the presence or absence of the fertilization membrane. The results are analyzed by standard statistical methods to yield a time-dependent median effective concentration (EC_{50}) (that is, the concentration of the test material that is effective in reducing fertilization success by 50 percent) after a specified period of exposure.

Sperm cell toxicity tests with two reference toxicants (silver nitrate and the pesticide endosulfan) showed that toxic responses were directly related to sperm exposure time. The 60-min EC_{50}s and 95 percent fiducial limits for green sea urchin (*Strongylocentrotus droebachiensis*) sperm were 76 (57 to 103) and 168 (99 to 285) μg/litre for silver and endosulfan, respectively. The 60-min EC_{50}s for sand dollar (*Dendraster excentricus*) sperm were 45 (34 to 61) and 147 (123 to 175) μg/litre for silver and endosulfan, respectively. Matrix bioassays using temperature, salinity, and toxicant concentration as variables showed that the toxic response to both silver and endosulfan generally increased with increasing temperature and decreasing salinity. One test series of silver and endosulfan in combination showed that toxicity was additive. Comparative sea urchin and sand dollar embryo tests (72 to 96 h) showed embryo sensitivity to be similar to sperm sensitivities with levels of effect (retardation of development) of 50 and 25 μg/litre, respectively, for silver and 253 and 158 μg/litre for endosulfan.

Additional development and refinement of this sperm cell toxicity test should yield a rapid and sensitive procedure for screening large numbers of toxicants in marine waters, aid in the prediction of ecological effects, and provide an additional tool for the biomonitoring of receiving water quality.

KEY WORDS: toxicity test, sperm, embryos, sea urchin, sand dollar, marine toxicity, silver, endosulfan, aquatic toxicology, hazard assessment.

Scientists charged with the responsibility of assessing, monitoring, and regulating the use of toxic substances are being increasingly challenged by

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the proliferation of chemicals for a wide range of societal applications. Biological screening techniques which can be conducted rapidly at the least cost and which retain maximum sensitivity are needed in conjunction with sensitive analytical chemical techniques. Various aspects of biological systems must be considered, including toxicity, mutagenicity, carcinogenicity, and teratogenicity. The currently popular Ames microbial mutagenesis test [*1–2*][2] is one example of a refined and powerful short-term test in which single-cell bacteria are used to predict the mutagenicity (and, to a large extent, the carcinogenicity) of many chemicals in higher animals. This test is not entirely precise or absolute, but it is a valuable screening test that is quick and practical and has the additional benefit of providing valuable insight into basic biological functions and relationships.

Short-term aquatic toxicity tests that are as powerful as the Ames test are presently lacking. Early life-stage and life-cycle tests are possible alternatives, but these may be very time-consuming and difficult to conduct and interpret. Enzyme tests are somewhat quicker and very sensitive, but discrete biochemical reactions are being analyzed instead of an integrated biological system.

The sperm cell is a functioning biological unit which has been the object of extensive research, especially in relation to sea urchins [*3–6*]. Sperm cell tests using a variety of methods have been used successfully with many toxicants, including metals [*7–10*], petroleum [*11–12*], organic compounds [*13*], and municipal wastewaters [*14–15*]. These studies have shown sperm cells to be sensitive to a variety of toxicants in short-term tests (that is, minutes) usually at or below concentrations causing mortality in standard 96-h fish or invertebrate toxicity tests.

Based on this information, we suggest that a standardized test procedure using sea urchin sperm cells be developed and used to provide a screening technique for the establishment of toxicity indexes for a wide array of chemical toxicants in marine waters. Toxicity indexes would be useful as general biological baseline data for the setting of priorities for further research, the biomonitoring of receiving waters, and regulatory action.

The purpose of this research was to investigate conditions for conducting toxicity tests with sea urchin and sand dollar sperm and to utilize this test to determine the toxicity of two distinctly different reference toxicants: a metal (silver) and an organochlorine pesticide (endosulfan).

Materials and Methods

All toxicity tests were conducted at the Fisheries Research Institute's Mobile Bioassay Laboratory located at the West Point Sewage Treatment Plant, Seattle, Wash.

[2]The italic numbers in brackets refer to the list of references appended to this paper.

Test Animals

Green sea urchins (*Strongylocentrotus droebachiensis*) were collected from a rocky breakwater in Elliott Bay, central Puget Sound, Wash., at a depth of 1 to 5 m below the mean lower low water. Animals for general use were held for up to 4 months in ambient flowing seawater and were supplied with macroalgae as a food source. Sand dollars (*Dendraster excentricus*) were collected from a sandy beach in southern Puget Sound from approximately the mean lower low water level and maintained in ambient flowing seawater on sand substrate for up to 5 months. Plankton and detritus in the ambient seawater and sand substrate were the only food supply.

Spawning and Gamete Quantification

The test animals were spawned separately by the potassium chloride (KCl) method [*16*] (0.5 to 1.0 ml 0.5 *M* KCl injected through the peristomal membrane into the coelomic cavity). Sperm were collected in 100-ml filtered (5-μm) seawater and kept in this concentrated condition until just prior to their use. Eggs were collected in 100 to 250-ml filtered seawater and washed two or three times before use. All gametes were kept cool in an ambient temperature seawater bath (Fig. 1).

Subsamples of sperm were counted by compound microscope and hemacytometer at ×400 after fixing in 10 percent acetic acid in seawater. Subsamples of eggs were counted using a dissecting microscope at ×10 to 20. Sperm were diluted to a final concentration of 50×10^6/ml, and eggs were diluted to 5×10^3/ml. Most tests used 0.1-ml aliquots of sperm solution in 25 ml of test solution with the subsequent addition of 1-ml aliquots of egg solution. This combination produced a final sperm-egg ratio of 1000/1 and dilution of the test solutions of 0.4 percent upon the addition of sperm and 4.0 percent during the addition of eggs.

Sperm Toxicity Test Procedure

Most sperm tests were conducted by adding 0.1-ml aliquots of sperm solution to 25-ml replicate (×2 to 4) toxicant and control solutions contained in 25 by 150-ml glass test tubes held in a circulating water bath of the desired temperature. After an appropriate exposure period (typically, 15 to 60 min), 1.0 ml of eggs was added to the sperm–toxicant solution and fertilization allowed to proceed for 15 min. The test tubes were then carefully decanted and the settled eggs poured into labeled vials containing 10 percent formalin in seawater for sample preservation and storage (Fig. 1). The term *sperm exposure time*, as used in this report, is defined as the time between the addition of sperm to the toxicant and the subsequent addition of eggs. The sperm (and eggs) were exposed to the toxicant for an additional period of time while fertilization (if any) took place. This "extra" period of exposure is unavoidable

but should remain constant between bioassays using the same sperm–egg contact time interval.

Each egg sample was later tabulated for percentage fertilization by resuspending the eggs in each vial, pouring the contents onto a grid-lined glass slide, and scoring 100 eggs for the presence or absence of the fertilization membrane. Partially formed membranes were tabulated as unfertilized eggs. Occasional broken membranes which otherwise appeared complete were counted as fertilized.

Embryo Toxicity Tests

Embryo tests were conducted in a series of 250-ml glass beakers each containing 100 ml of toxicant solution or control dilution water. Each concentration was duplicated. The bioassays were initiated by inoculating each container with approximately 5000 freshly fertilized eggs. Development was allowed to proceed for 72 or 96 h. Temperature control was maintained by an ambient temperature seawater bath. At 72 or 96 h the samples were fixed and stored in 10 percent formalin in seawater. Stirring or aeration of the test containers was not required. Mortality was assessed by comparing triplicate subsample counts of embryos from each container at 72 or 96 h with triplicate subsample counts from two extra initial control samples. Stages of embryonic development and abnormalities were assessed in subsamples of 100 embryos by using a compound microscope at ×40 and descriptions from the published literature [*13,17–19*].

Water Quality Control

The natural seawater used in most tests was pumped from Puget Sound from a depth of approximately 10 m with a cast-iron pump. All supply and distribution lines were polyvinyl chloride (PVC) plastic (conditioned in seawater for more than 3 years). The laboratory head tank was fiberglass. Prior to its use in tests, all natural seawater was filtered through a 5-μm cellulose filter cartridge and an activated carbon cartridge.

Salinity was adjusted by dilution with fresh tap water dechlorinated with activated carbon or deionized water. The temperature was controlled during the tests with either an ambient temperature seawater bath or a heating–cooling circulating freshwater bath. Any required pH adjustments of the toxicant or dilution water were made with gradual additions of either reagent-grade hydrochloric acid (HCl) or sodium hydroxide (NaOH) to the desired pH.

Toxicants

Silver test solutions were freshly prepared by the appropriate additions of 100 or 1000-mg/litre stock solutions of reagent-grade silver nitrate ($AgNO_3$) in distilled water to a measured volume of seawater. Each test solution was

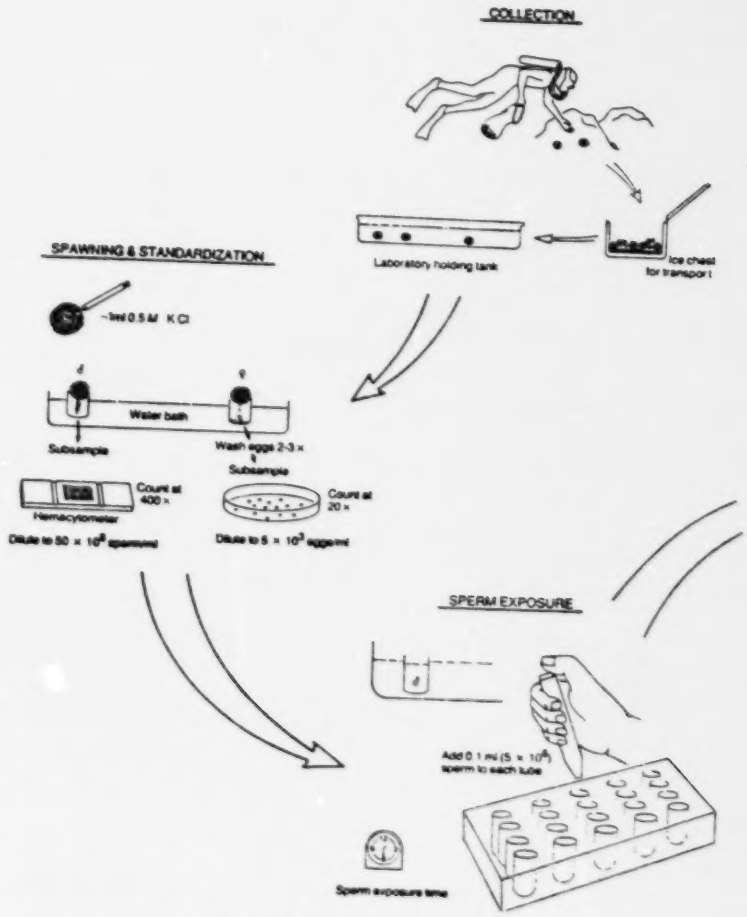
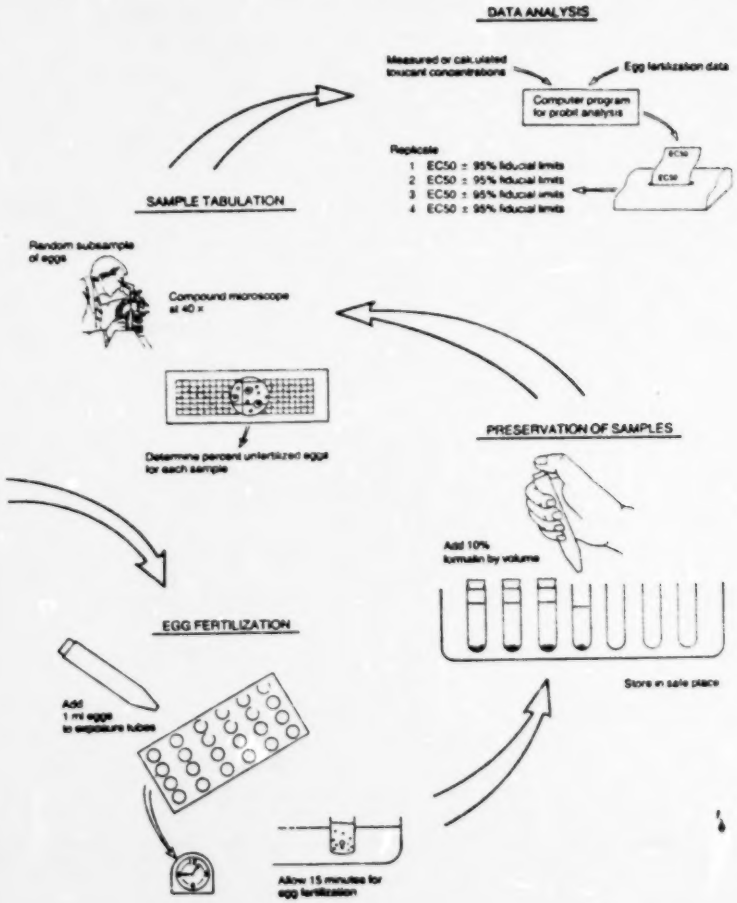


FIG. 1—*Sperm cell test flow diagram.*

then split into replicate 25-ml test samples and a sample for chemical analysis. This procedure was repeated until all the test concentrations were prepared with the desired number of replicates. Simultaneously, other test solutions were prepared in precisely the same manner using a certified commercial silver reference solution (1 ml = 1 mg as silver). Samples of these solutions were retained for chemical analysis in conjunction with the $AgNO_3$ toxicity test solutions to provide a standard curve for comparison of the bioassay test concentrations by least squares regression analysis. Sperm or embryo toxicity tests were then conducted as previously outlined.


DATA ANALYSIS
Measured or calculated toxicant concentrations
Egg fertilization data
Computer program for probit analysis
Replicate
1 EC50 ± 95% fiducial limits
2 EC50 ± 95% fiducial limits
3 EC50 ± 95% fiducial limits
4 EC50 ± 95% fiducial limits
SAMPLE TABULATION
Random subsample of eggs
Compound microscope at 40 ×
Determine percent unfertilized eggs for each sample
PRESERVATION OF SAMPLES
Add 10% formalin by volume
Store in safe place
EGG FERTILIZATION
Add 1 ml eggs to exposure tubes
Allow 15 minutes for egg fertilization

The replicate silver samples for chemical analysis were transported to the Fisheries Research Institute's Water Quality Laboratory, University of Washington, Seattle, Wash. Samples were analyzed with a Perkin-Elmer 303 atomic absorption spectrophotometer with DCRI concentration readout assembly and silver lamp within 6 h of the sample preparation. The lamp current was 10 mA; the slit was 4; the wave length was 328.1; the oxidant was air; and the fuel was acetylene.

Endosulfan stock solutions were prepared by adding technical-grade endosulfan [obtained from the U.S. Environmental Protection Agency (EPA) Laboratory, Corvallis, Ore.] in excess of its reported solubility (approximately 250 μg/litre) to prefiltered (0.45-μm) natural or artificial seawater. The stock solutions were filtered again after thorough mixing and then diluted to provide toxicant solutions of desired concentrations, which in turn were divided into replicate 25-ml test volumes and added to the test tubes. The toxicant solution from one of the tubes at each concentration was used only in the determination of the actual concentration of endosulfan at the initiation of each test. Similar procedures were followed for α-endosulfan, β-endosulfan, endosulfan ether, and endosulfan sulfate (obtained from FMC Corp., Middleport, N.Y., and EPA, Health Effects Research Laboratory (HERL), Research Triangle Park, N.C.).

Chemical analyses of endosulfan test solutions were performed as follows: the 25-ml water sample and approximately 10 ml of nanograde hexane were added to a 60-ml separating funnel equipped with a Teflon stopcock. The funnel was shaken vigorously, and after phase separation the hexane was drained into a 50-ml volumetric flask. The water sample was extracted a second time with an additional 10 ml of hexane, which was added to the first portion. The flask was brought to volume (which represented a 1:2 dilution) and approximately 3 to 4 g of anhydrous sodium sulfate were added. A portion of the extracted pesticide in hexane was transferred to glass tubes equipped with aluminum foil-lined caps and stored until analyzed by gas chromatography.

A Perkin-Elmer Model Sigma 1 gas chromatograph equipped with a 10 mC nickel-63 (^{63}Ni) electron capture detector was used for analysis. A glass column of 1.83-m by 2-mm inside diameter (ID) packed with 100/200 mesh Supelcoport coated with 3 percent SP-2100 was used at a column flow rate of 40 cc 95 percent argon (5 percent methane) per minute and detector flow rate of 65 cc 95 percent argon (5 percent methane) per minute. The temperatures of the injector, column, and detector were 245, 230, and 300°C, respectively.

Nominal silver and endosulfan concentrations varied from test to test but generally ranged from 10 to 400 μg/litre for silver and 25 to 600 μg/litre for endosulfan.

Sperm Test Data Analysis

Generally, each sperm test conducted under a specific set of conditions was replicated four times by using sperm from four separate males. This experimental design yielded four estimates of the 50 percent effective concentration (EC_{50}) per test condition, which took into consideration any variability due to differences in sperm quality and the counting-dilution error relating to sperm exposures.

All the sperm test responses (percentage of unfertilized eggs) to be used for EC_{50} concentrations were corrected for the natural responsiveness of the controls by Abbott's formula [*20*]

$$\text{Adjusted test response} = \frac{\text{Percent test response} - \text{percent control response}}{100 - \text{percent control response}} \times 100$$

The corrected responses were used for the EC_{50} calculations using the BMDO3S computer program for probit analysis with an attached FORTRAN program to calculate 95 percent fiducial limits for each EC_{50} [*21*]. Mean EC_{50}s and 95 percent fiducial limits were then calculated for each set of replicate EC_{50}s by calculating a weighted mean value of the individual EC_{50} estimates, using weights in inverse proportion to the variances [*22*]. All EC_{50}s and 95 percent fiducial limits presented in this paper were calculated using the weighted mean method. Individual replicate EC_{50}s and 95 percent fiducial limits are summarized elsewhere [*23*].

Results

Spawning Cycles

Sea urchins and sand dollars were monitored for sperm and egg production and for their viability to determine the seasonal availability of ripe animals for sperm tests. A sufficient number of green sea urchins held in ambient temperature (8 to 10°C) seawater spawned from 2 Feb. to 2 April 1979. Sea urchins held in water cooled several degrees below ambient temperature continued spawning until 8 June. Sand dollars produced sufficient gametes from 20 July to 6 Oct. 1979. The thermal conditioning of sand dollars was not attempted but should be possible by holding them at reduced water temperatures.

Sperm Tests

Physical Conditions—Sets of sperm tests were conducted with each species to determine the test limitations with respect to temperature, salinity, pH, dissolved oxygen, sperm/egg ratios, and sperm viability with time. Generally, sperm/egg ratios of 1000/1 were found to be satisfactory for sperm "exposure" periods up to 60 min in natural seawater under optimal conditions. Occasional tests with sperm exposure times >60 min were conducted with higher sperm/egg ratios. Practical limits for temperature, salinity, pH, and dissolved oxygen at a sperm/egg ratio of 1000/1 were determined (Table 1).

Toxicity Tests—Equivalent sperm tests with two reference toxicants were conducted with sea urchin and sand dollar sperm. These tests assessed the toxicity of silver and endosulfan to sperm cells under a variety of test conditions. The test conditions included sperm exposure time (15 to 120 min), pH (7.5, 8.0, 8.5), temperature (7, 12, 17°C), and salinity (24, 26, 28 parts per thousand). The EC_{50}s for each toxicant under each test condition were determined for sea urchin sperm (Table 2) and for sand dollar sperm (Table 3). The results of matrix tests with more than two variables can be represented graphically by three-dimensional computer graphic techniques, as illustrated for silver at three temperatures and three salinities (Fig. 2).

One hypothesis we wished to test was that sperm cells were more sensitive

TABLE 1—*Physical limitations of the sperm cell toxicity test.*[a]

Condition	Green Sea Urchin (*Strongylocentrotus droebachiensis*)	Sand Dollar (*Dendraster excentricus*)
Temperature	2 to 17°C	2 to 22°C
Salinity		
Natural seawater	≥28 ppt[b]	≥24 ppt
Artificial seawater	≥30 ppt	≥30 ppt
pH	7.5 to 8.5	7.0 to 8.5
Dissolved oxygen	. . .	0.8 mg/litre—saturated
Sperm viability time	~20 h	>6 h

[a] For sperm/egg ratio of 1000/1 and "exposure times" ≤60 min.
[b] ppt = parts per thousand.

to toxicants than were the eggs and would, therefore, make a better test tool. To test this hypothesis we exposed either sperm or eggs to silver or endosulfan prior to fertilization. The EC_{50}s (Table 4) clearly showed that the sperm cells were more sensitive, usually by a substantial margin. In two tests both sperm and eggs from sand dollars were exposed to either silver or endosulfan simultaneously before fertilization. In these cases the resulting EC_{50}s were

TABLE 2—*Toxicity of silver and endosulfan to sea urchin sperm.*

Test Variable[a]	EC_{50} and 95% Fiducial Limits, μg/litre	
	Silver	Endosulfan
Exposure time, min		
15	589 (368 to 942)	. . .
30	168 (108 to 262)	321 (241 to 427)
60	76 (57 to 103)	168 (99 to 285)
90	49 (30 to 82)	<66 . . .
120	. . .	164 (98 to 274)
pH		
7.5	247 (108 to 562)	99 (13 to 787)
8.0	273 (141 to 530)	358 (201 to 639)
8.5	237 (76 to 73[illegible]	766 (38 to 15 291)
Temperature, deg Celsius; salinity ppt[b] matrix		
7; 28	215 (151 to 306)	556 (297 to 1 040)
7; 26	112 (53 to 236)	358 (258 to 498)
7; 24	47 (28 to 78)	130 (42 to 400)
12; 28	110 (74 to 163)	637 (359 to 1 130)
12; 26	148 (85 to 258)	140 (72 to 273)
12; 24	130 (51 to 327)	<83 . . .
17; 28	38 (28 to 51)	460 (250 to 848)
17; 26	38 (23 to 62)	252 (132 to 482)
17; 24	20 (11 to 38)	<84 . . .

[a] General test conditions were temperature = 7.5 to 8.5°C; salinity = 27.7 to 29.1 ppt; pH = 7.8 to 8.0; sperm exposure time = 30 min, unless the condition was a test variable as specified in the table.
[b] ppt = parts per thousand.

TABLE 3—*Toxicity of silver and endosulfan to sand dollar sperm.*

Test Variable[a]	EC_{50} and 95% Fiducial Limits, μg/litre	
	Silver	Endosulfan
Exposure time, min		
15	177 (113 to 278)	. . .
30	119 (102 to 140)	156 (124 to 198)
60	45 (34 to 61)	147 (123 to 175)
90	24 (18 to 33)	103 (75 to 142)
120	. . .	80 (63 to 101)
pH		
7.5	49 (34 to 71)[b]	227 (200 to 258)
8.0	46 (32 to 67)[b]	264 (217 to 321)
8.5	38 (25 to 59)[b]	226 (192 to 265)
Temperature, deg Celsius; salinity, ppt[c] matrix		
7; 28	120 (68 to 212)	165 (133 to 204)
7; 26	105 (50 to 218)	<68 . . .
7; 24	66 (39 to 113)	107 (85 to 133)
12; 28	88 (58 to 132)	234 (168 to 326)
12; 26	78 (52 to 118)	115 (93 to 143)
12; 24	76 (32 to 179)	147 (96 to 225)
17; 28	66 (60 to 73)	238 (204 to 277)
17; 26	61 (42 to 90)	150 (124 to 181)
17; 24	41 (22 to 76)	201 (153 to 265)

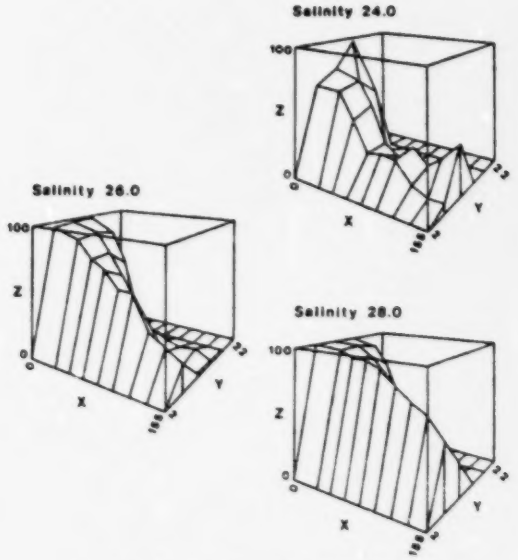
[a]General test conditions were temperature = 12.6 to 13.2°C; salinity = 28.0 ppt; pH = 8.0; sperm exposure time = 30 min, unless the condition was a test variable as specified in the table.
[b]Sperm exposure time = 60 min.
[c]ppt = parts per thousand.

not significantly different from the EC_{50}s for only sperm exposed to the same toxicant. This further indicated that the primary toxic action was on the sperm and not on the eggs which were added for the fertilization reaction.

A second hypothesis we tested was that the sperm test was as sensitive (or more sensitive) as longer term embryo tests. In each case, 72-h sand dollar or 96-h sea urchin embryo development tests proved to be only as sensitive as or less sensitive than (based on 50 percent retardation of development) 60-min sperm exposure tests (Tables 5 and 6) [*24–31*].

One series of sand dollar sperm exposures tested the relative toxicity of four chemical forms of endosulfan (α, β, sulfate, and ether). The relative toxicity of these four forms as determined by sperm tests was $\beta >$ sulfate $> \alpha >$ ether, with 60-min EC_{50}s and 95 percent fiducial limits of 60 (50 to 71), 212 (170 to 266), 269 (230 to 316), and 579 (510 to 658) μg/litre, respectively.

One additional set of sand dollar sperm exposures tested the interactive toxicity of silver and endosulfan in combination. A response surface plot of the results (Fig. 3) suggested that the toxicity of silver and endosulfan in combination was additive.



x—axis is silver concentration, 0 to 155 μg/litre
y—axis is temperature, 2 to 22°C
z—axis is percent fertilization, 0 to 100 percent

FIG. 2—*Surface response plots of the toxicity of silver to sand dollar sperm with varying salinity and temperature. Each data point used for constructing the plots represents the average percentage of egg fertilization of four replicates.*

TABLE 4—*Comparative sensitivities of sea urchin and sand dollar gametes to silver and endosulfan.*

Gamete Exposed to Toxicant[a]	EC_{50} and 95% Fiducial Limits, μg/litre	
	Silver	Endosulfan
Sea urchin		
Sperm only	225 to 250	168 (99 to 285)
Eggs only	8 000 to 16 000[b]	>751
Sand dollar		
Sperm only	44 (29 to 67)	316 (274 to 364)
Eggs only	>255	322 (209 to 495)
Sperm and eggs	51 (31 to 85)	225 (155 to 326)

[a]General test conditions were temperature = 7.7 to 13.0°C; salinity = 28.0 parts per thousand; pH = 8.0; and gamete exposure time = 60 min.
[b]Exposure time = 30 min.

TABLE 5—*Comparative toxicity of silver to echinoid sperm, embryos, and other aquatic animals.*

Test Organism	Exposure Time	Water Type*	EC_{50} or LC_{50}, µg/litre
Sea urchin sperm (*Strongylocentrotus droebachiensis*)	15 min	SW	589
	30 min	SW	168
	60 min	SW	76
Sand dollar sperm (*Dendraster excentricus*)	15 min	SW	177
	30 min	SW	119
	60 min	SW	45
Sea urchin embryos (*S. droebachiensis*)	96 h	SW	100 (>50% retardation)
Sand dollar embryos (*D. excentricus*)	72 h	SW	50 (>50% retardation)
Crab zoea (*Carcinus maenas*) [*24*]	96 h	SW	10 to 100
Daphnia (*Daphnia magna*) [*25*]	1 to 2 h	FW	5.1 (immobilization)
Oyster embryo (*Crassostrea virginica*) [*26*]	48 h	SW	5.8
Rainbow trout (*Salmo gairdneri*) [*27–28*]	96 h	FW	6.5 to 28.8

*FW = fresh water; SW = seawater.

TABLE 6—*Comparative toxicity of endosulfan to echinoid sperm, embryos, and other aquatic animals.*

Test Organism	Exposure Time	Water Type*	EC_{50} or LC_{50}, µg/litre
Sea urchin sperm (*Strongylocentrotus droebachiensis*)	30 min	SW	321
	60 min	SW	168
	90 min	SW	<66
Sand dollar sperm (*Dendraster excentricus*)	30 min	SW	156
	60 min	SW	147
	90 min	SW	103
Sea urchin embryos (*S. droebachiensis*)	96 h	SW	>253 (>50% retardation)
Sand dollar embryos (*D. excentricus*)	72 h	SW	106 to 188 (>50% retardation)
Daphnia (*Daphnia magna*) [*29*]	48 h	FW	200
Amphipod (*Gammarus lacustris*) [*30*]	24 h	FW	9.2
Rainbow trout (*Salmo gairdneri*) [*31*]	24 h	FW	3.2 to 13

*FW = fresh water; SW = seawater.

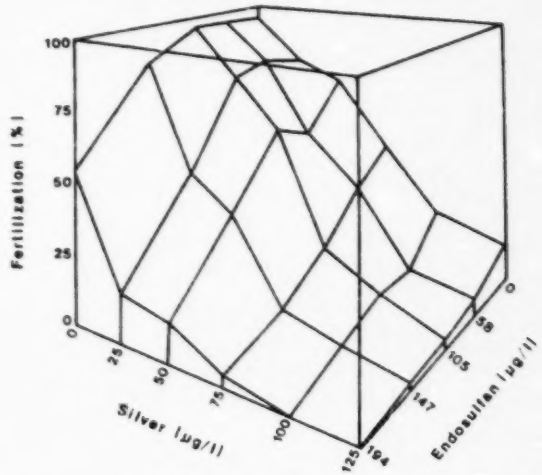


FIG. 3—*Surface response plot of the toxicity to sand dollar sperm of silver and endosulfan in combination. Each data point used for constructing the plot represents the average percentage of egg fertilization of four replicates.*

Discussion

The sperm cell toxicity test is a quick and sensitive test for some toxicants in marine waters. Sperm tests conducted using the tentative methods outlined in this paper have shown that sea urchin and sand dollar sperm sensitivity to silver and endosulfan was similar to the sensitivity of 72 to 96-h echinoid embryo tests and within 1 order of magnitude of the sensitivity of many acute tests with other aquatic animals (Tables 5 and 6). Sea urchin sperm have also proved sensitive to chlorine and bromine in the 2 to 20 µg/litre (total residual oxidant) range [*15*] and to an urban–industrial sewage effluent at concentrations five to seven times less than 96-h median lethal concentrations (LC_{50}s) for marine fishes and invertebrates [*15,32*].

The sperm test has most of the advantages of both static and flow-through tests with few of the disadvantages. Being a static test, it utilizes single batches of dilution water with constant, closely defined water quality characteristics. Temperature fluctuations are avoided by using a constant-temperature water bath. The short duration of the sperm test minimizes the loss or degradation of the toxicant in the test containers and facilitates the analysis of the toxicant. The test response (percentage of unfertilized eggs) is easily quantified in an objective manner and the results easily analyzed with accepted standard toxicity test statistical methodology. Adult animals are easily obtained in coastal marine waters and maintained in the laboratory. The adult animals are rarely harmed by the KCl spawning treatment, which allows them to be used repeatedly (two to three times with about 30-day rest-

ing intervals) as sources of gametes or returned unharmed to their natural environment.

This test should prove to be especially useful for monitoring the variable toxicity of chemicals which change form or degrade rapidly with time after they are introduced into seawater, or complex wastewater samples which normally receive extensive chemical characterization but no biological assessment. The sperm test should also prove to be a valuable biomonitoring procedure for receiving water quality as well as a standardized laboratory procedure. Its use in a matrix arrangement with multiple test conditions has been illustrated in this paper. The synergistic or antagonistic interactions of combinations of toxicants can also be analyzed in a similar matrix design.

The sperm test described in this paper needs further refinements to optimize its usefulness and repeatability. The tests reported here generally used a ratio of 1000 sperm to 1 egg for both species. However, some of our results suggest that a 1000/1 sperm to egg ratio may not be high enough for green sea urchin sperm, while 250 sperm to 1 egg may be a satisfactory ratio for sand dollars. The optimum sperm/egg ratios should be determined for each species. Control fertilization for most tests described in this paper was ≥90 percent. Occasional tests which utilized sperm exposure times greater than 60 min resulted in control fertilization of substantially less than 90 percent. While the values of sperm response to the toxicant concentrations can be easily corrected for the "natural responsiveness" of the controls using Abbott's formula [*20*], the resolution of the sperm responses to the toxicant decreases as control fertilization decreases. However, there is nothing magical about 100 percent (or ≥90 percent) control fertilization. Indeed, the maximum sensitivity of the sperm test is probably realized at a point where the sperm/egg ratio yields less than 100 percent control fertilization, since this would ensure that a surplus of sperm did not exist in the test tubes and, therefore, *any* toxic effect on the sperm in the toxicant concentrations should be resolved in relation to the controls.

Variability between tests should be reduced to narrow the fiducial limits of the EC_{50}s and facilitate comparisons between treatments or species. Variability can be reduced by more accurate enumeration of the initial sperm and egg densities so that the final dilutions to predetermined standard densities will yield minimal variability between individual sperm and egg samples. Fiducial limits may be tightened by increasing the number of test replicates per treatment, the optimum number probably being a compromise between the number required for a solid statistical base, on the one hand, and those realistically possible based on the time and funds available, on the other.

Disposable test tubes (with caps) should help to refine the sperm test by reducing the time and effort involved in washing test tubes and storage vials. The eggs would be preserved by adding formalin to each test tube after the egg fertilization period and subsequently capping each tube until the eggs could be counted. This procedure would also eliminate any possibility of contamination from previous tests or from cleaning agents.

We hypothesize that the sensitivity of sperm from many species of sea urchins (and sand dollars) to toxic substances is essentially equal. Our research found that green sea urchin and sand dollar sperm are roughly of equal sensitivity to both silver and endosulfan based on generally overlapping 95 percent fiducial limits of the pooled EC_{50}s for both species for the time-concentration tests (Tables 2 and 3). These comparisons are admittedly rough, as the test methods were continually undergoing refinement and the optimum sperm/egg ratio may be different for each species. Additional replicates (more than four) may be necessary to yield reasonably tight estimates of the EC_{50}.

Improved availability of ripe test animals is desirable. Presently, by maintaining green sea urchins in cooled seawater during their normal spawn-out period, gametes can be obtained from approximately February to June. Sand dollars appear to be normally ripe from June to October and possibly through November, if they are thermally conditioned. December and January may represent periods of time when gametes are not available from these two species. Testing should be extended to other species of sea urchins to close this gap. The purple sea urchin (*Strongylocentrotus purpuratus*) has been widely used for experimental biology purposes along the Pacific Coast of the United States. This species is plentiful on the open coast and has a prolonged spawning cycle compared to *S. droebachiensis* [*33*], with active spawning beginning in December [*34*]. The red urchin (*S. franciscanus*) is also abundant on the Pacific Coast. Should sperm of these species all prove equally sensitive to various toxicants, it would then be interesting to compare our results with similar testing of other U.S. and foreign species.

An extended corollary of this hypothesis is that sperm cells from many other marine animals exhibiting external fertilization may show the same sensitivity in this test procedure as sea urchin sperm. Oysters (*Crassostrea gigas*) should provide a valuable comparison since they can be thermally conditioned to spawn out of season and the embryos serve as the basis of the Washington State receiving water quality bioassay [*35*].

The use and application of the sperm test can be further extended by conducting comparative 96-h toxicity tests with selected species of marine fishes and invertebrates. Such comparisons will relate the sensitivity of the sperm test to existing toxicity data and help validate this test for use in refining water quality criteria and for assessment of potential ecological impacts.

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